

Overexpression of a Laccase with Dye Decolorization Activity from *Bacillus* sp. Induced in *Escherichia coli*

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Keywords

Laccase · *Bacillus* sp. · *Escherichia coli* · Enzyme · Dye decolorization

Abstract

Laccases from bacteria have been widely studied in the past 2 decades due to the higher growth rate of bacteria and their excellent thermal and alkaline pH stability. In this study, a novel laccase gene was cloned from *Bacillus* sp., analyzed, and functionally expressed in *Escherichia coli*. The laccase was highly induced in the *E. coli* expression system with a maximum intracellular activity of 16 U mg⁻¹ protein. The optimal temperature and pH of the purified laccase were 40°C and 4.6, respectively, when ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonate]) was used as the substrate. The purified laccase showed high stability in the pH range of 3.0–9.0, and retained more than 70% of its activity after 24 h of incubation at 40°C with a pH value of 9.0. Furthermore, the enzyme exhibited extremely high temperature and ion metal tolerance. The half-life of the purified laccase at 70°C was 15.9 h. The purified laccase could efficiently decolorize 3 chemical dyes, especially in the presence of ABTS as a mediator. The high production of this laccase in *E. coli* and exceptional characteristics of the recombinant enzyme protein make it a promising candidate for industrial applications.

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Introduction

The *CotA* gene from *Bacillus* encoding for a 65-kDa protein has been identified as copper-dependent laccase (EC 1.10.3.2) [Hullo et al., 2001]. The *CotA* protein exhibits similar characteristics of multi-copper oxidases such as those from fungi, insects, and plants, which contain 4 histidine-rich copper-binding sites corresponding to 1 type-1, 1 type-2, and a pair of type-3 copper ions [Duraó et al., 2006; Hakulinen et al., 2002]. Laccase can catalyze the oxidation of a broad variety of chemicals or substrates, such as phenolic and nonphenolic compounds, polycyclic aromatic hydrocarbons, and chemical dyes, due to its wide substrate specificity [Liu et al., 2012; Minussi et al., 2002; Morozova et al., 2007]. Therefore, laccase has been proposed as a potential candidate for the development of environmental and industrial applications in the biobleaching of pulp, industrial wastewater treatment, soil bioremediation, textile dyes, lignin degradation, and organic synthesis [Couto and Herrera, 2006; Cristóvão et al., 2011; Morozova et al., 2007]. The potential applications of laccases urge us to find more novel laccase producers and evaluate their biochemical characteristics and practical applications [Madhavi and Lele, 2009; Minussi et al., 2002].

Among the various strategies found for producing laccases in microorganisms in nature, fungi, especially white

atggcactagaaaaatttgcagatgaactgccgattattgaaacctccagccgcagaagaaatcagacggcagcacgtattatgaagtc	90
M A L E K F A D E L P I I E T L Q P Q K K S D G S T Y Y E V	30
acgatgaaggaatgctttcacaagctgcaccgcgatctcccgccaccggctgtgggggtataacggtttatttcccggcccgcagatc	180
T M K E C F H K L H R D L P P T R L W G Y N G L F P G P T I	60
gacgtgaaccaagatgagaacgtctatattaatggatgaatgaccttccggataagcattttctgccgtggatcataaccattcacat	270
D V N Q D E N V Y I K W M N D L P D K H F L P V D H T I H H	90
tcagaaagcggccatcaggaacccgatgtcaaacccgtcgtccatttacacggaggagcaacgcccgccggacagcgacggctaccggaa	360
S E S G H Q E P D V K T V V 2 H L H G G A T P P D S D G Y P E	120
gcatggttcacaaaggattttaagaaacgggtccttattttgagaagaggatataccattatccaaacaaacagcgcggggctctgtta	450
A W F T K D F K E T G P Y F E K E V Y H Y P N K Q R G A L L	150
tggtatcacgatcacgccatggcgattacaggctcaatgtgtacgccgggcttgccgggatgtatatcatccgcgagcgaagaaaag	540
W Y 3 H D H A M A I T R L N V Y A G L A G M Y I I R E R K E K	180
cagctgaagcttccctccggagagtagcagctaccgcttatgattttggaccgcacgttaaatgacgacggttcttgtcttatccgagc	630
Q L K L P S G E Y D V P L M I L D R T L N D D G S L S Y P S	210
ggacctgtaacccttccgaaacgctgcccgacccttccatcgctcccggttttttggcgaaataccattctcgtcaatggtaaagcgtgg	720
G P A <u>N P S</u> E T L P D P S I V P F F C G N T I L V N G K A W	240
ccgtatatggaagtgaaccgggacatatcgtttccgcaccttaatgcctccaatacaagaacattttctctcctcgtcaataacggc	810
P Y M E V E P R T Y R F R I L <u>N A S</u> N T R T F S L S L N N G	270
gggaagtttatccaaatcggttctgacggcggactgcttcccgcctctgtcaaacacaatctatcagcttagcccggcagagcgggat	900
G K F I Q I G S D G G L L P R S V K T Q S I S L A P A E R Y	300
gatgtgctcatgatttctccgcctttgacggagaacatatcattttaacgaacggcaccggctgcgggggcagcggcatccggataca	990
D V L I D F S A F D G E H I I L T <u>N G T</u> G C G G D A D P D T	330
gacgccaatgtgatgaattccgcgtcacaaaaccgctgaaggagcagacacaagccggaagcctaaatatctttcaacaatgcctaat	1080
D A N V M Q F R V T K P L K G A D T S R K P K Y L S T M P <u>N</u>	360
gtgacatcaaagagaatacacaatatacaggacgcttaaacacacaatacgaagacaaatacggccggcccgttttgacgctgaataac	1170
<u>V T</u> S K R I H N I R T L K L T N T Q D K Y G R P V L T L N N	390
aagcgatggcatgatcctgtgacagaagcggcgaagctcggcacaacggaaatctggtcgattatcaaccgatggggggcaccatccg	1260
K R W H D P V T E A P K L G T T E I W S I I N P M G G T 1 H P	420
atacacctgcacctgttttcttccaaatccttgaccggcgccttttgacttagagcgttataacaaatcggcgacattgtgtataca	1350
2 I H L H L V S F Q I L D R R P F D L E R Y N K F G D I V Y T	450
ggccccgccgtcccgcctcctccaagtgagaaagctggaagataaccgtgcaggcgcactcgggagaggtcatcagaatcgcgggcact	1440
G P A V P P P P S E K G W K D T V Q A H S G E V I R I A A T	480
ttcgtccttacagcggacgctacgtatggcactgtcatatttttagagcacgaagattacgacatgatgagaccgatggacgtcacggat	1530
F A P Y S G R Y V W 3 H C H I L E H E D Y D M M R P M D V T D	510
aagcagtaa	1539
K Q -	512

Fig. 1. Full-length cDNA sequence and its deduced amino acid sequence of LacA4. Four N-linked glycosylation sites (N-X-T) are indicated by underlining and 4 histidine-rich copper binding domains are indicated by boxes. The numbers indicate the type of copper: numbers 1, 2, and 3 indicate the type 1, 2, and 3 copper centers.

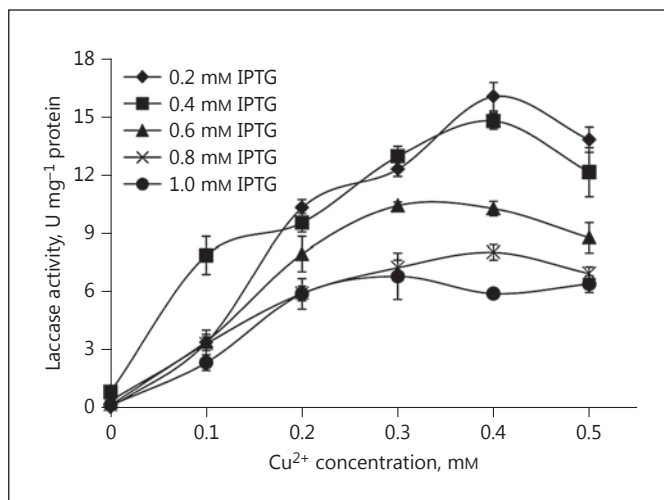


Fig. 2. Laccase overexpression in *E. coli* BL21 (DE3) was highly activated by copper ions. Laccase protein was induced for 3 h using IPTG in the presence of different concentrations of copper ions when OD₆₀₀ of the recombinant *E. coli* BL21 strain reached 0.6–0.8. Data are the mean ± SD ($n = 3$).

rot fungi, have been the best candidates for secreting extracellular laccase [Levin et al., 2004]. However, most fungi need a long time to grow and produce laccases that are unstable under high temperatures, high salt, and alkalinity stress [Hildén et al., 2009]. These drawbacks result in low efficiency and prohibitive costs in industrial processes [Santhanam et al., 2011]. Compared to fungal laccases, the prokaryotic laccases exhibit the outstanding advantages of high thermal and alkaline pH stability [Santhanam et al., 2011]. Furthermore, bacteria usually have higher growth rates and it is easier to enhance their activity, selectivity, and expression levels through genetic manipulation [Gronenberg et al., 2013].

Escherichia coli is one of the most effective expression systems for the production of recombinant proteins [Makrides, 1996]. However, ligninolytic enzymes like laccase are generally regarded as the most difficult proteins to express in bacterial systems [Bulter et al., 2003]. Yano et al. [2009] tried to express recombinant laccases in several conventional expression systems, including *E. coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris*, but the laccase gene cannot be successfully expressed in the overexpression system. The laccase from *Haloferax volcanii* was successfully overexpressed in *E. coli*, but no laccase activity was detected [Uthandi et al., 2010]. The undetectable laccase activity may be partially due to the fact that the laccase from a halophilic archaeon *H. volcanii* is halophilic and after expression it needs to be dialyzed against

buffer containing 2 M of NaCl to activate the enzyme [Uthandi et al., 2012]. It has also been reported that the haloarchaeal proteins, glycoproteins, and enzymes with elaborate metal clusters are all extremely difficult to overexpress in conventional expression systems, such as *E. coli* and *S. cerevisiae* [Madzak et al., 2005; Uthandi et al., 2010]. Furthermore, most of the recombinant laccases were kept in cytoplasm or formed insoluble intracellular fractions, even though the laccase activity was detected in the *E. coli* expression system [Santhanam et al., 2011]. In this study, we report the cloning and successful heterologous production of the thermo-alkali stable laccase of *Bacillus* sp. in *E. coli*. The overexpression system was highly induced to produce soluble and intracellular laccase in the presence of copper ion (Cu²⁺). This is a promising approach to producing laccase for industrial applications.

Results

Cloning and Analysis of Bacillus sp. A4 Laccase cDNA

The laccase gene of this *Bacillus* strain contained an open reading frame of 1,539 bp, which encodes 512 amino acids with a predicated molecular mass of 58.06 kDa and a theoretical isoelectric point of 6.556. The sequence of the laccase has been deposited into the NCBI GenBank with the accession number KX674736. The laccase contained 4 potential N-glycosylation sites (Asn-Xaa-Ser/Thr), at positions 214, 256, 318, and 360 of the deduced protein and 10 histidine residues, 1 cysteine residue, and 1 methionine residue (Fig. 1), which was similar to most known *Bacillus* laccases [Hullo et al., 2001; Lu et al., 2013].

Production of Recombinant Laccase in E. coli

In this study the recombinant laccase was not highly induced by a range of isopropyl-β-D-thiogalactoside (IPTG) concentrations, but it was markedly activated in the presence of Cu²⁺ (Fig. 2). After 3 h of incubation, the highest intracellular laccase activity induced by IPTG 0.4 mM was 0.84 U mg⁻¹ of protein, while it was 16 U mg⁻¹ of protein in the presence of 0.2 mM of IPTG and 0.4 mM of Cu²⁺ (Fig. 2).

Biochemical Characteristics of the Recombinant Laccase

The recombinant laccase produced by *E. coli* BL21 (DE3) was subjected to sodium dodecyl sulfate (SDS)-PAGE and Western blot analyses. The sample showed 2 protein bands with molecular masses of about 87.5 and 68 kDa, but the zymogram analysis of the laccase only ex-

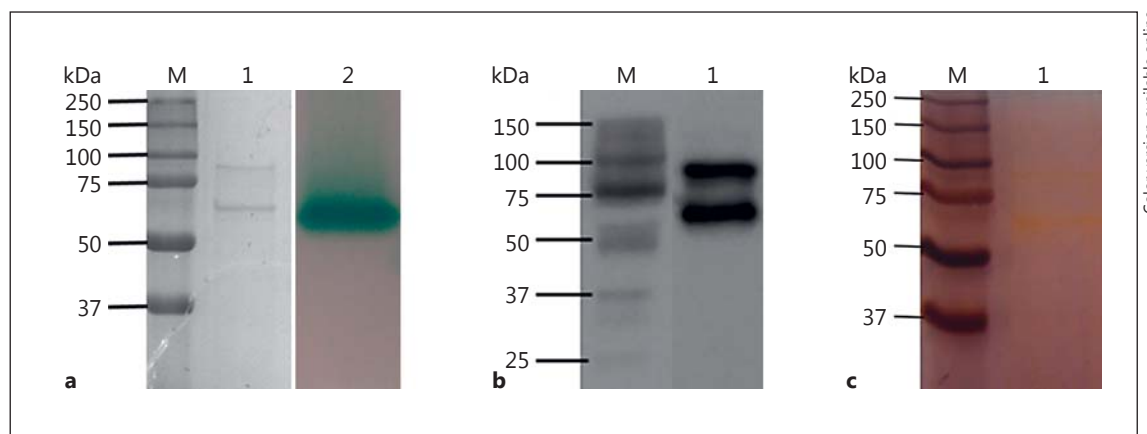


Fig. 3. Electrophoretic analysis of the purified laccase. **a** SDS-PAGE was stained with CBBR-250. Lane M, protein marker; lane 1, purified laccase was denatured at 100°C for 10 min; lane 2, zymogram was stained with 1 mM of ABTS. **b** Western blot analysis using His-tag antibody. Lane M, protein marker; lane 1, purified laccase. **c** Glycosylation analysis of purified enzyme using PAS-silver stain. Lane 1, purified laccase was denatured at 100°C for 10 min.

Table 1. Substrate specificity of the purified laccase

Substrate	λ_{\max} , nm	ϵ , $M^{-1} \text{ cm}^{-1}$	K_m , μM	k_{cat} , s^{-1}	k_{cat}/K_m , $s^{-1} M^{-1}$
ABTS	420	36,000	31.2	72.6	2.32×10^6
2,6-DMP	469	49,600	161.2	45.4	2.82×10^5
Guaiacol	465	12,100	542.8	29.0	5.35×10^4

hibited 1 band of approximately 68 kDa (Fig. 3a). The 2 bands could be immunologically recognized in Western blot analysis using the anti-His tag antibody as the primary antibody (Fig. 3b). Furthermore, the 2 proteins were both glycosylated in the potential N-glycosylation sites of the protein according to SDS-PAGE analysis by silver staining (Fig. 3c).

The purified recombinant laccase showed an extensive pH range for oxidizing different substrates (Fig. 4a). The optimal pH values for catalyzing ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonate]), guaiacol, and 2,6-DMP (2,6-dimethoxyphenol) of the laccase were 4.6, 7.4, and 7.8, respectively (Fig. 4a). The recombinant laccase was observed to be stable in the pH range of 3.0–9.0, and retained more than 70% of its activity after 24 h of incubation at 40°C with a pH value of 9.0 (Fig. 4b). The highest activity for oxidizing ABTS was found at 40°C; the relative laccase activities were 88.9, 82.3, 63.5, 50.6, and 26.8% at 30, 50, 60, 70, and 80°C as compared to that at the optimal temperature, respectively (Fig. 5a). The thermostable range test of the enzyme exhibited that the re-

combinant laccase was relatively stable between 30 and 40°C, and still retained more than 70 and 50% of its activity after 24 h of incubation at 50 and 60°C, respectively (Fig. 5b). Furthermore, more than 80% of the activity of the enzyme was present at 60–70°C for 6 h, and the half-life of the enzyme was approximately 15.9 h at 70°C (Fig. 5b).

The kinetic parameters of the recombinant laccase were evaluated at optimal pH and temperature levels using ABTS, 2,6-DMP, and guaiacol as the substrates (Table 1). The purified enzyme showed the highest specificity for ABTS with the lowest K_m value of 31.2 μM compared to the other 2 substrates, which had corresponding K_m values of 161.2 and 542.8 μM for 2,6-DMP and guaiacol, respectively (Table 1). The k_{cat} values of the enzyme for ABTS, 2,6-DMP, and guaiacol were 76.2, 45.4, and 29.0 s^{-1} , respectively, and the k_{cat}/K_m values were 2.32×10^6 , 2.82×10^5 , and 5.35×10^4 $s^{-1} M^{-1}$, respectively (Table 1). Furthermore, the enzyme was highly stable in the presence of ion metals and 5–10% organic solvents (online suppl. Fig. S1a, b; for all online suppl. material,

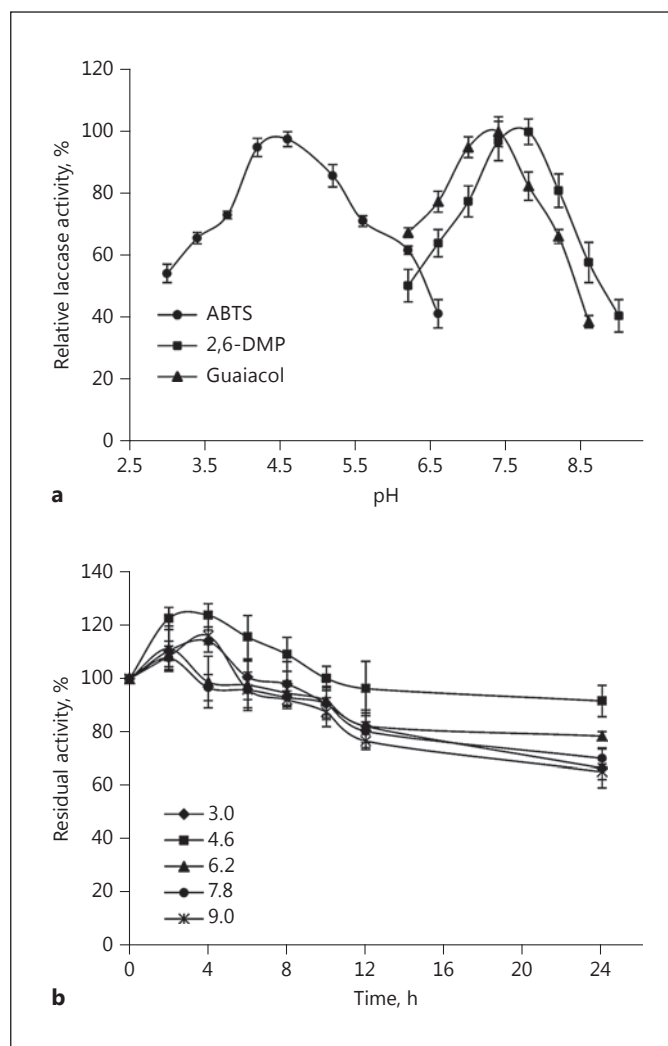


Fig. 4. Effect of pH on the activity (a) and stability (b) of the purified laccase enzyme at 40°C. **a** ABTS, pH 3.0–6.8; 2,6-DMP and guaiacol, pH 6.4–9.0. **b** Residual laccase activity was measured after incubation at different pH values for 0–24 h using the ABTS as a substrate. Data are the mean \pm SD ($n = 3$).

see www.karger.com/doi/10.1159/000478859). Most ion metals did not markedly impact on the activity of the recombinant laccase except the Mn^{2+} ions, which resulted in a reduction of 40% of its activity in the presence of 1.0 mM of Mn^{2+} (online suppl. Fig. S1a). However, like most laccases from fungi and bacteria, it was significantly decreased by some inhibitors, such as SDS, ethylenediaminetetraacetic acid (EDTA), and L-cysteine, which resulted in reductions of 71.2, 90.8, and 100% (totally lost activity) of its activity in the presence of 1.0 mM of SDS, EDTA, and L-cysteine, respectively (online suppl. Fig. S1c).

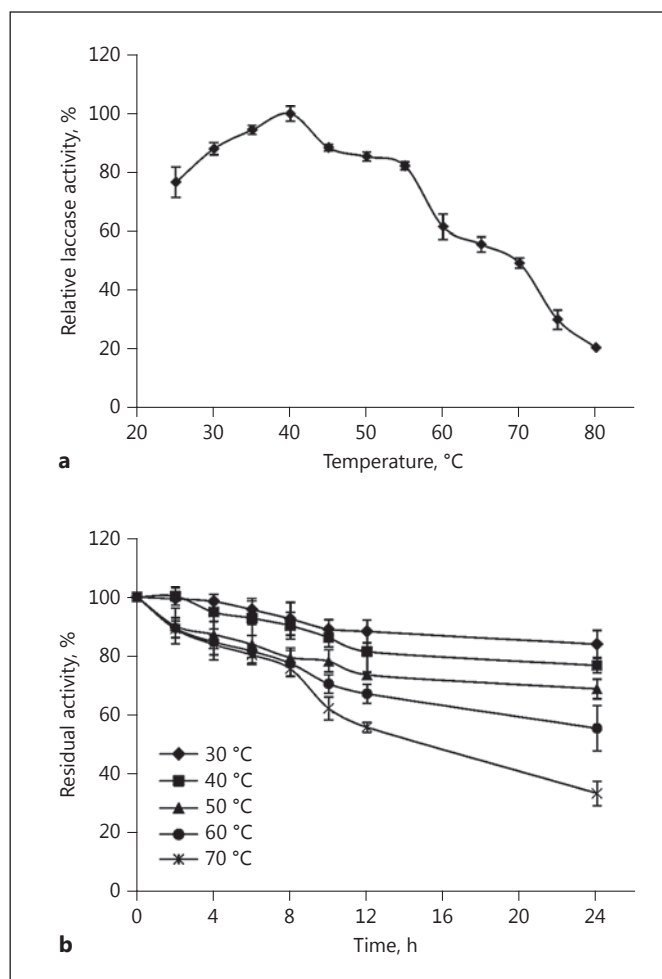


Fig. 5. Effect of temperature on the activity (a) and stability (b) of the purified laccase enzyme at pH 4.6. **a** Laccase was measured at different temperatures (25–80°C). **b** Residual laccase activity was measured after incubation at 30–70°C for 0–24 h using the ABTS as a substrate. Data are the mean \pm SD ($n = 3$).

Dye Decolorization

In this study, we evaluated the decolorization ability of the purified recombinant laccase produced by *E. coli* using an azo dye (Congo red; CR) and 2 triarylmethane dyes (Coomassie brilliant blue R-250 [CBBR-250] and crystal violet [CV]). The purified laccase efficiently decolorized different chemical dyes with a range decolorization extent of 75–99%, especially in the presence of a redox mediator (ABTS; Fig. 6). As the soluble dyes were decolorized some small particles were formed, which were indicated by arrows in the wall and bottom of the test tubes (Fig. 6a–c). After 10 h of incubation, the decolorization extents were 75.7, 87.0, and 82.9% for CBBR-250, CR, and CV, respectively, when only the purified laccase was added into the

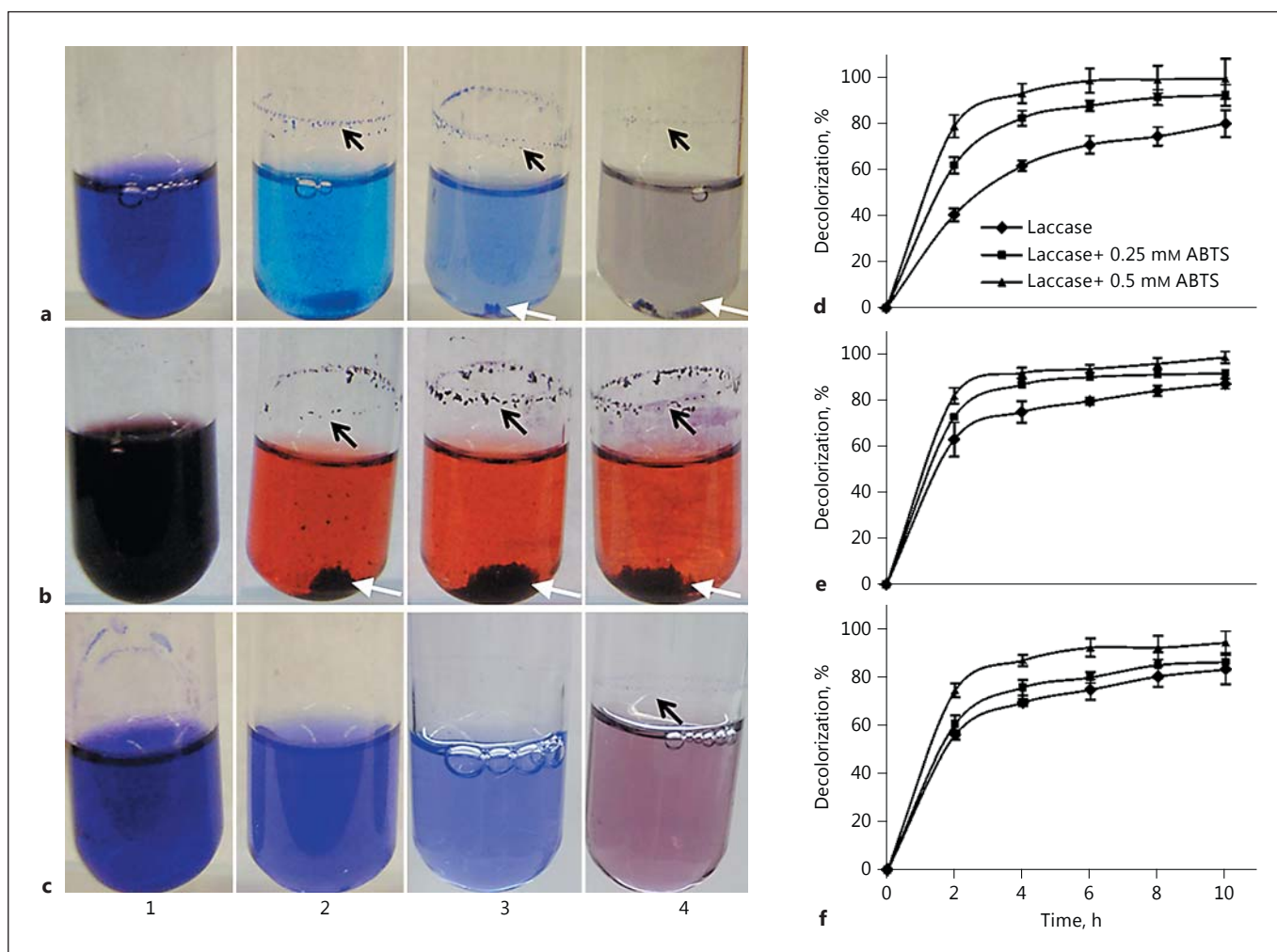


Fig. 6. a–c Effects of purified laccase on decolorization of CBBR-250 dye, CR, and CV. 1, dye; 2, laccase + dye; 3, laccase + 0.25 mM ABTS + dye; 4, laccase + 0.5 mM ABTS + dye. Decolorization of CBBR-250 dye (d), CR (e), and CV (f) by the purified laccase at 40°C for 0–10 h. Data are the mean \pm SD ($n = 3$).

0.02% chemical dye solutions. However, the decolorization was more efficient in the presence of purified laccase and 0.5 mM of ABTS together, with corresponding decolorization extents of 99.1, 98.5, and 94.1%, respectively (Fig. 6d–f).

Discussion

Laccases have been widely applied in the textile industry, bioremediation and biodegradation, the paper and pulp industry, and the chemical industry due to its oxidation of both the toxic and nontoxic substrates [Shekher et al., 2011]. The features of laccases from bacteria have

been widely studied due to their high thermal and alkaline pH stability and easy genetic manipulation. However, most bacterial laccases show drawbacks of low production yields, which have impeded their further application in industry process [Couto and Herrera, 2006; Sharma et al., 2007]. In this study, a gene encoding for a laccase from *Bacillus* sp. was cloned and successfully expressed in *E. coli*. The laccase cDNA was inserted into pET-21a vector, which is a tightly regulated and highly IPTG-inducible bacterial expression vector that allows the expression of the protein of interest in *E. coli* cells and can be easily purified by a C-terminal His-tag [Sharrocks, 1994]. The results showed that the recombinant laccase was not highly induced by IPTG, but markedly activated by IPTG and

Cu²⁺ with a maximum activity of 16 U mg⁻¹ of protein (Fig. 2, 3a). It was reported that laccases as well as other ligninolytic enzymes are very difficult to express in bacterial systems [Bulter et al., 2003]. Yano et al. [2009] tried to express recombinant laccase from *Lentinula edodes* into *E. coli*, *S. cerevisiae*, and *P. pastoris*, but no laccase activity was detected in the presence of the inducer, even if Cu²⁺ was added. A laccase gene from *B. licheniformis* was successfully expressed in *P. pastoris* and the maximum activity induced by 0.1 mM of Cu²⁺ was 227.9 U L⁻¹ on the 7th day [Lu et al., 2013]. The laccase-like phenol oxidase from *Streptomyces griseus* was expressed with an *E. coli* host-vector system and the recombinant protein was highly induced by 1.0 mM of IPTG and 10 μM of Cu²⁺, and the highest activity was 8 U mg⁻¹ of protein on the 3rd day [Endo et al., 2003]. Furthermore, the laccase can also be secreted into the medium in the presence of 0.2 mM of IPTG and 0.4 mM of Cu²⁺, and the highest activity reached 281.5 U L⁻¹ after 7 days of induction (online suppl. Fig. S2), which was 1.24 and 2.68 times higher than the activity of *Trametes* sp. 48424 laccase (104.5 U L⁻¹) and *B. licheniformis* LS04 laccase (227.9 U L⁻¹) overexpressed in *P. pastoris*, respectively [Fan et al., 2011; Lu et al., 2013]. Considering the lack of signal peptide in the laccase protein, the accumulation of laccase in the culture medium could be mainly attributed to the cell lysis, which was followed by the release of the enzymes into the medium. Subsequently, the laccase activity was decreased along with the decrease of OD₆₀₀ (online suppl. Fig. S2), which may be due to the secretion of proteases caused by cell lysis [Wang et al., 2010].

The recombinant plasmid pET-21a/Lac expressed 2 proteins with a molecular mass of 87.5 and 68 kDa in the *E. coli* expression system by SDS-PAGE and Western blot analysis (Fig. 3a, b). Berna and Bernier [1997] demonstrated that 2 isoforms can be synthesized from a single gene since tobaccos were transformed with the wheat *gf-2.8* gene due to the posttranscriptional modifications of this protein. The zymogram analysis showed that the recombinant protein with a higher molecular mass did not have detectable activity, while the lower molecular mass protein showed a high laccase activity (Fig. 3a), which may also be relative to posttranslational processing. Furthermore, glycosylation is a form of posttranslational processing that is probably responsible for the size and stability of the protein [Roth et al., 2010; Solá and Griebenow, 2009]. This is why the protein molecular weight was much bigger than the predicted molecular mass and the laccases from other *Bacillus* strains [Martins et al., 2002; Telke et al., 2011].

It has been reported that most laccases from *Bacillus* species showed alkaline resistance and were highly stable at a pH range of 7.0–10.0 [Guan et al., 2014; Lu et al., 2012]. In this study, the recombinant laccase oxidized ABTS in acidic medium with an optimum pH value of 4.6, which was the same as most of the fungal laccases [Madhavi and Lele, 2009] and some bacterial laccases [Guan et al., 2014; Lu et al., 2012]. The optimal pH values for oxidizing guaiacol and 2,6-DMP in alkaline conditions were 7.4 and 7.8, respectively, like the laccases from other *Bacillus* species [Guan et al., 2014; Ye et al., 2010]. The enzyme showed the highest activity at 40°C and retained high activity at 30–55°C (Fig. 5a), which is similar to the findings of many researchers who demonstrated that the optimal temperatures of laccases are between 30 and 80°C [Lu et al., 2012; Sondhi et al., 2014; Telke et al., 2011]. Furthermore, the recombinant laccase showed a high thermostability with a half-life of about 15.9 h at 70°C. The half-life of inactivation at 70°C was much higher than some other *Bacillus* strains, such as 6.9 h at 70°C in LS04 [Lu et al., 2013] and 3.5 h at 65°C in DSM 27 [Reiss et al., 2011], and was similar to those from other *Bacillus* strains [Guan et al., 2014; Sondhi et al., 2014]. The higher stability of the enzyme might be adapted to cope with the industrial process requirements due to longer stability under high temperatures.

The purified laccase showed the lowest K_m values (31.2 μM) for oxidizing ABTS when compared to the other tested substrates, and the K_m value was also significantly lower than other *Bacillus* strains, which fell in the range of 44–1,404 μM [Lu et al., 2013; Mohammadian et al., 2010; Sondhi et al., 2014], indicating that the laccase from *Bacillus* sp. A4 has a much higher affinity for ABTS substrate. The higher k_{cat} and k_{cat}/K_m values showed by the purified enzyme indicated that it also has higher catalytic efficiency than other laccases [Lee et al., 2012; Lu et al., 2007]. Furthermore, the purified laccase showed excellent ion metal tolerance by retaining a higher stability in the presence of all of the tested ion metals except Mn²⁺ (online suppl. Fig. S1A). This is similar to the laccase from *Cerrena* sp., which was significantly affected by Mn²⁺ rather than the other ion metals [Yang et al., 2015]. The purified laccase can be gently induced by a low concentration of organic solvent (5% methanol and ethanol), which is consistent with the recombinant laccase from *B. licheniformis* [Lu et al., 2013]. However, like most laccases from fungi and bacteria, it was significantly decreased by some inhibitors, such as SDS, L-cysteine, and EDTA, which may affect the type I copper binding site of laccase by chelating with Cu²⁺ [Johannes and Majcherczyk, 2000; Prins et al., 2015].

Chemical dyes have been frequently used in the textile processing industry, in food technology, and in paper and drugs production [Rafii et al., 1997; Waring and Hallas, 2013], and have posed a serious threat to our environment [Forgacs et al., 2004; Singh and Arora, 2011]. The application of laccase in dye degradation has been widely studied by many researchers over the last few decades [Couto and Herrera, 2006; Nguyen and Juang, 2013]. In this study, the purified laccase was able to efficiently decolorize 3 chemical dyes, especially in the presence of ABTS as a mediator (Fig. 6). It has been reported that the presence of redox mediators, such as ABTS or syringaldehyde, is very important for the decolorization of various dyes, and significantly increases the decolorization extent of laccase [Dubé et al., 2008; Hou et al., 2004]. Furthermore, the decolorization extents were 10–21% higher than other laccases from *Bacillus* [Guan et al., 2014; Zhang et al., 2012].

In conclusion, a novel laccase gene from *Bacillus* sp. A4 was cloned and successfully expressed in *E. coli* BL 21 (DE3). Expression in *E. coli* was highly induced by Cu²⁺ according to the results of SDS-PAGE and Western blot. The recombinant protein with a higher molecular mass did not have detectable activity, while the lower molecular mass protein showed a high laccase activity by zymogram analysis. Furthermore, the recombinant laccase showed some interesting characteristics, such as a high thermostability, broad pH range, excellent ion metals tolerance, and high efficiency in dye decolorization. These characteristics make this laccase a potential candidate for industrial applications.

Experimental Procedures

Materials and Strains

Guaiacol, 2,6-DMP, ABTS, CBBR-250, CR, and CV were purchased from Sigma-Aldrich (St. Louis, MO, USA). Genomic DNA Mini Kit, Gel/PCR DNA Fragments Extraction Kit, and Plasmid Mini Prep Kit were purchased from FroggaBio (North York, ON, Canada). *Taq* DNA polymerase, pJET1.2 cloning vector, and IPTG were obtained from Thermo Fisher Scientific (Hudson, NH, USA). His TALON™ Gravity Column Purification Kit was obtained from Clontech (Palo Alto, CA, USA). The anti-His tag and goat anti-mouse IgG HRP antibody were purchased from Bio-Rad (Hercules, CA, USA). The Western Lightning Chemiluminescence Reagent Plus was obtained from PerkinElmer Life Sciences (Boston, MA, USA). All other chemicals and reagents were analytical grade.

The high-yield laccase-producing strain identified as *Bacillus* sp. A4 was isolated previously from forest soil (Thunder Bay, ON, Canada) in our laboratory (GeneBank No. KX665584) [Guo et al., 2017a]. Briefly, about 5 g of soil sample was added to 50 mL of sterile water; the mixture was agitated for 30 min at 200 rpm and

37°C, and 100 µL of the suspension was spread on Luria-Bertani (LB) medium containing 0.5 mM of guaiacol. Colonies forming reddish brown zones were selected and purified. The A4 strain was selected for further study because it showed the largest reddish brown zone and the highest laccase activity. The purified *Bacillus* sp. A4 strain was cultured in LB medium at 37°C with agitation at 200 rpm and stored at –70°C in a freezer. The *E. coli* DH5α and BL21 (DE3) were also stored at –70°C in our laboratory.

Cloning of the Laccase Gene and Sequence Analysis

For isolating the total DNA, *Bacillus* sp. was grown overnight in LB medium at 37°C with agitation at 200 rpm. One milliliter of culture was collected for DNA extraction using the Bacteria DNA kit. The laccase gene was amplified by PCR with forward primer 5'-GAATTCATGGCACTAGAAAAATTTGCAATG-3' and reverse primer 5'-GTCGACCTGCTTATCCGTGACGTC-CATCG-3'; the recognition sites of *EcoRI* and *Sall* are underlined. The PCR product was purified using the Gel Extraction Kit, and cloned into pJET1.2-T vector and transformed into *E. coli* DH5α. The plasmid DNA was then extracted for sequencing. The cloned laccase gene was digested with *EcoRI* and *Sall* and ligated with corresponding sites of the digested pET-21a vector. The final expression plasmid was constructed as pET-21a/Lac, which includes a 6-His tag at the C-terminus of the laccase gene.

The nucleotide sequence and the molecular weight and theoretical isoelectric point of deduced protein of the laccase gene were manually edited and analyzed with EidtSeq Module of DNASTar software (DNASTAR, UK). The conserved domains of the laccase protein were predicted by conserved domains in NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The N-glycosylation sites were predicted by NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>). The signal peptide cleavage site was identified by SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>).

Heterologous Expression of the Laccase Gene in *E. coli*

The pET-21a/Lac plasmid DNA was transformed into *E. coli* BL21 (DE3) by the heat shock method at 42°C for 90 s. Positive clones were selected on an LB medium plate containing 100 µg mL⁻¹ of ampicillin. In the induction experiments, colonies picked from the plate were inoculated with 2 mL of LB medium containing 100 µg mL⁻¹ of ampicillin (LB/amp) and incubated overnight at 37°C with agitation at 200 rpm. The next morning, *E. coli* BL21 (DE3)/pET-21a/Lac was inoculated in the fresh LB/amp medium in a 1:50 dilution and grown until the OD₆₀₀ reached 0.6–0.8. Subsequently, IPTG was added to a final concentration of 0.2, 0.4, 0.6, 0.8, and 1.0 mM in the presence of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM Cu²⁺, respectively, under the same culture condition. Then, cells from 1 mL of cultures of each treatment were collected by centrifugation at 12,000 g for 15 min at 4°C. The cell pellets were resuspended in 0.1 M of potassium phosphate buffer (pH 7.4) containing 50 mM of KCl and sonicated at a resonance of 40 kHz at 4°C for 2 min using the “duty cycle” control, which consisted of 10 s of working time and a 10-s interval for cooling down the samples on ice. The cell extract was obtained by centrifuging at 12,000 g for 20 min as crude enzymes and kept at 4°C for activity measurement.

For the purification of laccase protein, the enzyme produced in the presence of 0.2 mM of IPTG and 0.4 mM of Cu²⁺ was used due to the highest laccase activity. The purification was performed us-

ing the His TALON™ Gravity Column Purification Kit according to the manufacturer's instructions (Clontech, Mountain View, CA, USA). Briefly, about 0.5 g of cell pellets were resuspended in 10 mL of His TALON-xTractor buffer containing 5 µL of DNase I (Bio-Rad). The mixture was incubated on ice with intermittent mixing for 15 min and centrifuged for 20 min at 10,000 g at 4 °C. The His-tagged recombinant protein in the supernatant was purified by affinity chromatography using a gravity-flow column (Clontech) and eluted with phosphate buffer containing 150 mM of imidazole. The protein concentration was determined according to the method of Bradford [1976]. The purified protein was used for downstream biochemical characteristics and dye decolorization analysis.

Determination of Laccase Activity

Laccase activity was recorded at 40 °C by measuring the oxidized compounds of guaiacol in 0.1 M of Tris-HCl buffer (pH 7.4) at 465 nm ($\epsilon_{465} = 12,100 \text{ M}^{-1} \text{ cm}^{-1}$), 2,6-DMP in 0.1 M of Tris-HCl buffer (pH 7.8) at 469 nm ($\epsilon_{469} = 49,600 \text{ M}^{-1} \text{ cm}^{-1}$), and ABTS in 0.1 M of citrate buffer (pH 4.6) at 420 nm ($\epsilon_{420} = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$), according to the methods of Kittl et al. [2012], Mekmouche et al. [2014], and Guo et al. [2017b]. The absorbance was determined using a Microplate Spectrophotometer (Epoch, BioTek Instruments Inc., Winooski, VT, USA). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute. Kinetic constants were measured by varying substrate concentrations of ABTS (20–2,000 µM), 2,6-DMP (50–2,000 µM), and guaiacol (2–2,000 µM) at their optimal pH values according to the method of Kittl et al. [2012].

Gel Electrophoresis and Western Blot Analysis

To determine the molecular weight of the laccase from this strain, 15 µL of the purified enzyme was run in duplicate along with a standard protein marker using a 12% (w/v) polyacrylamide gel with an 8% stacking gel according to the method of Guo et al. [2017a]. After electrophoresis, the gel was divided into 2 parts. One part was stained with CBBR-250 to visualize the protein. The other part was immersed in 1% Triton-X 100 for 30 min to remove the SDS, the protein bands with laccase activity were visualized by soaking the gel in 0.1 M of citrate buffer (pH 4.6) containing 1.0 mM of ABTS. Glycosylation of the purified laccase was detected by silver staining according to the description of Gradilone et al. [1998]. Briefly, after SDS-PAGE electrophoresis, gels were fixed in 10% acetic acid (v/v) and 30% ethanol 3 times for 30 min each and incubated with 1% periodic acid (v/v) and Schiff reagent, respectively. The gels were then stained with 0.2% silver nitrate (w/v) and visualized by 2.3% sodium carbonate (w/v).

The purified laccase was used for Western blot analysis. After separation by SDS-PAGE, the proteins were transferred onto a PVDF membrane at 80 V for 2 h. The membranes were incubated with 1:1,000 anti-His tag antibody for 1 h at room temperature, followed by incubation with 1:2,000 goat anti-mouse IgG for 1 h at room temperature. The protein bands were then visualized using the Western Lightning Chemiluminescence Reagent Plus (Boston, MA, USA).

Effects of pH and Temperature on Recombinant Laccase Activity and Stability

The effects of pH on recombinant laccase activity towards guaiacol, 2,6-DMP, and ABTS were measured in 0.1 M of citrate buffer

(pH 3.0–6.6) and 0.1 M of Tris-HCl buffer (pH 6.2–9.0) at 40 °C. The residual activity was determined to evaluate the enzyme activity against pH after 24 h incubation in pH 3.0, 4.6, 6.2, 7.8, and 9.0 at 40 °C, respectively. The optimal temperature of the recombinant laccase was determined over the temperature range of 25–80 °C using ABTS as the substrate in 0.1 M citrate buffer (pH 4.6). The thermostability was assayed at 30–70 °C by incubating the recombinant laccase in 0.1 M citrate buffer (pH 4.6) for 24 h and the reaction was started by the addition of ABTS.

Effects of Metal Ions, Organic Solvents, and Inhibitors on Recombinant Laccase Activity

The effects of different metal ions, organic solvents, and inhibitors on laccase activity were determined at 40 °C with ABTS as the substrate in 0.1 M citrate buffer (pH 4.6). To measure the effects of metal ions, recombinant laccase was assayed in the presence of 1.0 or 5.0 mM LiCl, CdCl₂, ZnCl₂, CuCl₂, MgCl₂, CaCl₂, BaCl₂, AlCl₃, and CoCl₂, respectively. The effects of organic solvents (methanol, ethanol, acetone, and dimethyl sulfoxide) on the activity of laccase were measured by individually adding 5, 10, and 20% (v/v) of them into the reaction mixture. The influences of SDS, L-cysteine, and EDTA on laccase were examined by adding them to a final concentration of 0.1 and 1.0 mM, respectively.

Decolorization of Dyes

Decolorization of 0.02% (w/v) chemical dyes was carried out at 40 °C with 5 U of purified laccase in 5 mL of 0.1 M citrate buffer (pH 4.6) with agitation at 200 rpm. To evaluate the effect of ABTS on the dye decolorization, ABTS was added to a final concentration of 0.25 or 0.5 mM to initiate the enzyme reaction. The decolorization abilities were determined by recording spectrophotometrically the decrease of absorbance of CBBR-250 dye (563 nm), CR (643 nm), and CV (655 nm). The absorbance was recorded every 2 h and the images were taken after 24 h of incubation. The decolorization (%) was calculated as: (initial absorbance – absorbance after reaction)/initial absorbance × 100. The controls were treated equally without the addition of enzyme.

Statistical Analysis

All the experiments were performed in triplicate, and the results are presented as the mean ± SD. Statistical analysis was carried out by 1-way analysis of variance using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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